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A THRESHOLD SIZE FOR METAMORPHOSIS IN THE TOBACCO HORNWORM, *MANDUCA SEXTA* (L.)

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There exists convincing evidence that the onset of metamorphosis is accompanied by a decrease in the titer of juvenile hormone (JH) (Wigglesworth, 1970; Gilbert and King, 1973). The *corpora allata* (CA) cease to secrete JH during the final larval instar in most species of insects (Patel and Madhavan, 1969; de Wilde, de Kort and de Loof, 1971; Nijhout and Williams, 1974b) or during the penultimate instar in certain hemimetabolous insects (Ozeki, 1965; Johnson and Hill, 1973). The ensuing decline in the hemolymph titer of JH is the immediate cause of metamorphosis. Many studies have focused on this aspect of the endocrine control of insect metamorphosis.

While the role of JH titer decline in metamorphosis is well established, the mechanism controlling the timing of this event is not well understood. Classical experiments by Wigglesworth (1970) and Fukuda (1944) have demonstrated that the CA do not simply "count the instars" and shut off at a predetermined stage. It is therefore likely that timing of metamorphosis somehow relates to the stage of development that the larva has attained. A clear distinction should be made at the outset between factors which determine whether or not a molt shall proceed and those which determine the character of the molt. It is the latter factors that the present paper is concerned with. In its simplest form the problem can be stated as follows: on the basis of what type of information does a larva decide in which instar its CA are to be turned off? Recent studies by Riddiford (1970a, b) and Riddiford and Truman (1972) have demonstrated the existence of a "program" for postembryonic development in *Pyrrhocoris apterus*, *Oncopeltus fasciatus* and *Hyalophora cecropia* which determines the instar during which the CA will be inactivated. This program can be disrupted by an application of JH during late embryonic life. Animals treated in this way hatch and grow as normal larvae but fail to metamorphose. Instead, they may undergo a number of supernumerary larval molts and/or transform into larval-adult or larval-pupal intermediates due to the fact that their CA do not turn off during what is normally the final larval instar.

Allegret (1964) has shown that in the wax moth, *Galleria mellonella*, the "program" for the onset of metamorphosis is flexible and can be modified during postembryonic life. By isolating larvae at various stages of development on protein-free beeswax, he found that a larva becomes committed to metamorphose during the presumptive penultimate larval instar. The decision to metamorphose is apparently made at the outset of this instar but can still be modified by the amount of growth that occurs early in the instar. Unless a larva is allowed to grow

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for at least 24 hours during the presumptive penultimate instar, the CA remain active and normal larval molting continues indefinitely. Experimental alteration of the pattern of growth provides a powerful tool for the analysis of the processes that control molting and metamorphosis (Nijhout and Williams, 1974a, b). In the present paper I will demonstrate that factors associated with the somatic size of larval *Manduca sexta* play a decisive role in controlling the number of larval instars and hence the onset of metamorphosis.

Materials and Methods

Manduca sexta larvae were reared in individual containers as described by Truman (1972) at 25° C under a 12L : 12D photoperiod. Measurements of head capsule size were made by means of a calibrated ocular micrometer mounted in a dissecting microscope. The head capsule size was measured at its greatest width, at the level of the most anterior larval ocelli. Weights of larvae "at the time of molting" refer to individuals taken 12–24 hours prior to ecdysis at the time that their old head capsule had slipped forward and formed a muzzle over the developing head capsule of the new instar.

Results

Parameters of growth of Manduca *larvae*

Under the standard rearing conditions described above, *Manduca* larvae grow at a uniform rate and, without exception, undergo 4 larval molts before pupating. In soft-bodied larvae, such as those of *Manduca*, no striking changes in size occur when the larva molts from one instar to the next, and all somatic growth is confined to the intermolt period. For a given instar the maximum potential size that a larva can attain is most accurately estimated from the dimensions of a sclerotized part of its body. The head capsule is usually used for this purpose as it is one of the few parts that does not change in dimensions during the intermolt. Figure 1 is a plot of the weight of larvae at the time of the molt *vs.* the head capsule width of the succeeding instar. The dimensions of both parameters vary according to Dyar's rule (Dyar, 1890), *i.e.*, there is a constant growth ratio (1.6 for the head capsule width and 5.7 for the weight at the time of the molt) from instar to instar. Hence, the points show a good approximation to a straight line in a double logarithmic plot. This relationship has also been demonstrated in *Ostrinia nubilalis* by Beck (1950).

In order to determine whether a functional relationship exists between the weight that a larva achieves at the time of the molt and size of the new head capsule, I starved 4th instar larvae at various weights so that the molt to the 5th instar occurred at a subnormal weight. Figure 2 is a plot of the weight at which these starved larvae molted and the head capsule size of the resulting 5th instar. It is evident that a continuous range of 5th instar head capsule sizes can be produced by altering the weight at which the molt to this instar occurs.

The results of this experiment suggest that the mass of a larva at the time of the molt is instrumental in determining the size of the new head capsule, and hence, the potential size of the new instar. Figure 2 shows that the relationship

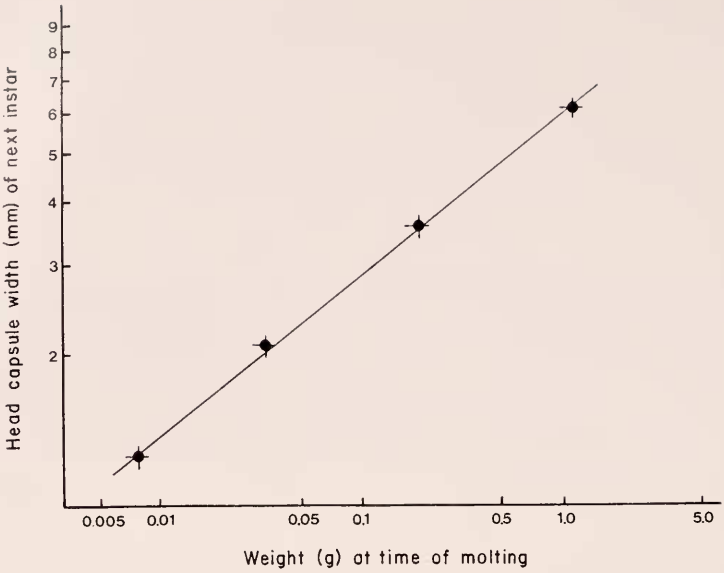

FIGURE 1. Double logarithmic plot of the weight of larvae at the time of molting *vs.* the head capsule width of the new instar for the 2nd through 5th larval instars of *Manduca sexta*. Each point represents the mean of 40 larvae. The bars indicate standard deviations.

between the weight at the time of the molt and the head capsule width of 5th instar larvae, derived from starved 4th instars, deviates significantly from the curve in Figure 1. This discrepancy is of interest and will be investigated in a future communication.

Effect of somatic size on the nature of the subsequent molt

In *Manduca,* and presumably most species of insects, metamorphosis does not occur until a certain minimum, species-specific size is attained. In analogy to the situation in *Galleria* (Allegret, 1964) it should be possible to delay or inhibit metamorphosis by preventing a larva from attaining its full potential size. In view of the fact that 90% of the growth of a larva occurs during the 5th (final) instar, I chose that stage for experimental manipulation. To prevent these larvae from attaining their full potential size, I starved them at various weights and observed the nature of the subsequent molt. Figure 3 illustrates the percent survival of final instar larvae as a function of the weight at starvation. When starvation was begun at a weight less than 3 g, few larvae survived to the next molt. Observations on larvae that died indicated that death was most likely due to dehydration. Indeed, when larvae were allowed to feed on a small block of 2% agar they survived several days longer than those that had no access to water. When these starved 5th instar larvae molted, they did not all pupate. Figure 3 also shows that when larvae weighing less than 4 g were starved, many molted into non-viable larval-pupal intermediates. The pattern of larval and pupal cuticle of these intermediates was identical to that obtained after application of JH

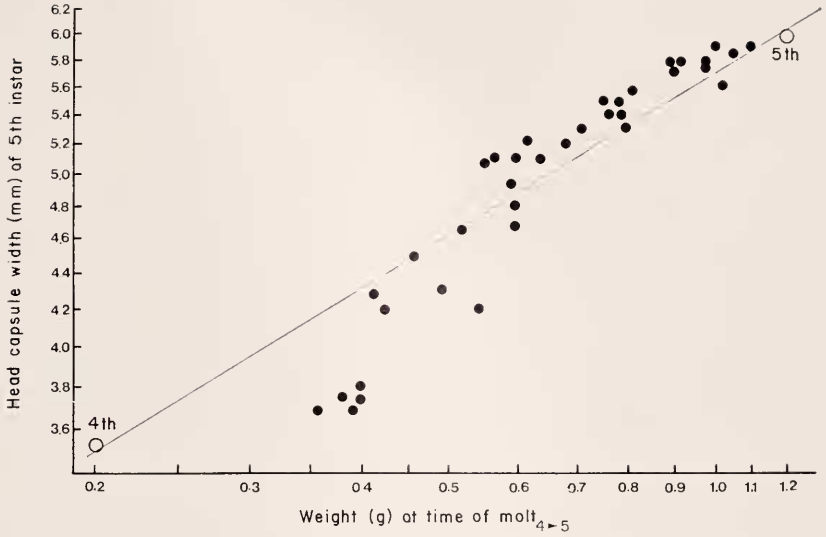

FIGURE 2. Plot of the weight at the time of molting of 4th instar larvae, starved at various weights, *vs.* the head capsule width of the resulting 5th instar larvae. The mean values for normal 4th and 5th instar larvae are indicated by the large open circles. The curve connecting these points is taken from Figure 1.

(Truman, Riddiford and Safranek, 1974). When larvae were starved at weights greater than 4 g, all eventually transformed into small but otherwise normal pupae. It was impossible to obtain viable additional larval instars by simple starvation of a final instar larva.

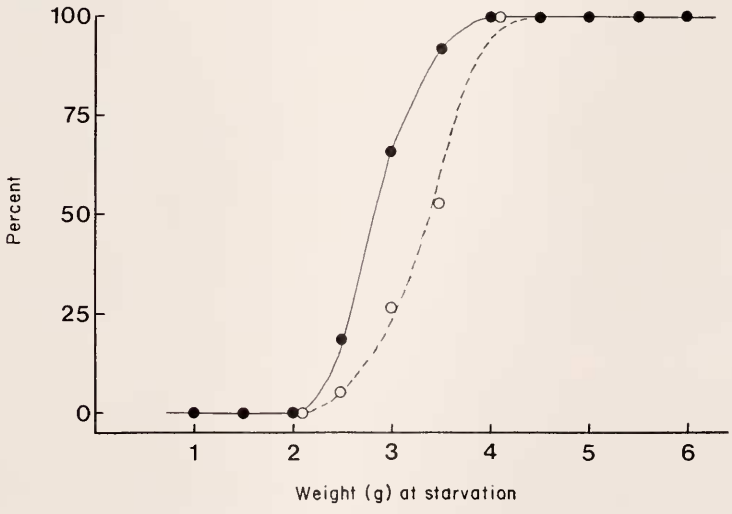

FIGURE 3. Filled circles, percent of 5th instar larvae that survive to molt after starvation various weights. Open circles represent percent surviving 5th instar larvae that molt to normal pupae. The remainder formed inviable larval-pupal intermediates.

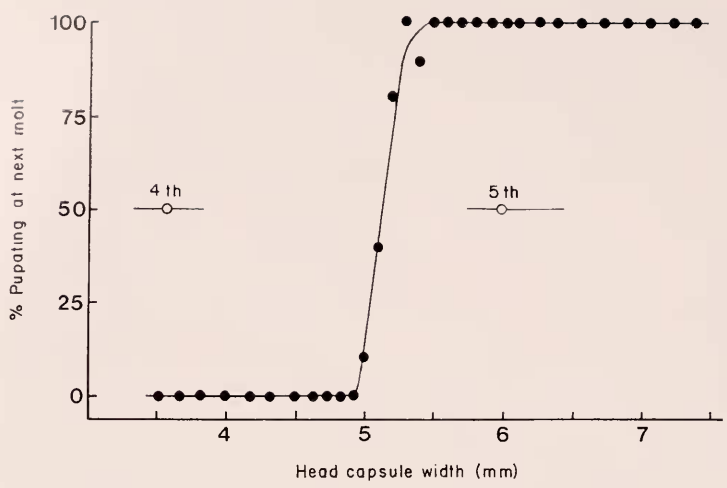

FIGURE 4. Percent of larvae with various head capsule sizes that will pupate at the succeeding molt. The mean (range) head capsule size of normal 4th and 5th instar larvae is indicated. Head capsule sizes were experimentally altered by starving 4th instar larvae at various weights. For detailed explanation, see text. Each point represents 9–25 larvae.

One variable that was not altered by these starvation experiments was the size of the exoskeleton of the larvae since the initial size of all individuals was identical. To determine whether the initial size of a 5th instar *Manduca* larva had any effect on the subsequent fate of the individual, I produced 5th instar larvae of various sizes as described under section 1 of results.

Figure 4 illustrates the fate of 5th instar larvae with various head capsule sizes. A sharp transition in the nature of the subsequent molt occurred at head capsule sizes intermediate between those of normal 4th and 5th instar larvae. The midpoint of this transition was at a head capsule size of 5.1 mm. Larvae with head capsules larger than 5.4 mm formed normal pupae. Those with head capsules measuring less than 5.0 mm always molted into an additional larval instar. The 6th instar larvae produced in this way were quite variable in size but were generally larger than a normal 5th instar. Those 6th instars derived from 5th with head capsules close to the threshold size of 5.1 mm were quite enormous. The head capsule sizes of these 6th instars were also included in Figure 4.

In three cases I obtained 6th instar larvae that were still of subthreshold size. This was due to lethargic feeding and slow growth. These individuals molted into a 7th instar before pupating. The head capsule sizes of these animals are listed in Table I.

In order to determine whether the threshold size depended on the size of the penultimate (4th) larval instar (*cf.* Allegret, 1964), I starved 3rd instar larvae at various weights thus inducing them to form 4th instars of various initial sizes (Figure 5). Fourth instar larvae with head capsules smaller than 3.2 mm (average width 2.95 mm) were selected for further observation. These larvae were allowed to resume feeding and proceeded to molt to 5th instars with a large range of head capsule sizes. Again, the fate of these 5th instar larvae depended only on the

TABLE I

Head capsule sizes (mm) of larvae starved as 4th instars which proceeded to form a 5th, 6th and 7th instar.

Larva	Instar number			
	4	5	6	7
1	—	4.19	5.15	7.10
2	—	4.36	4.80	6.40
3	—	4.38	5.00	6.10
Normal mean	3.55	5.95	—	—

size of their head capsule. Figure 6B shows that the transition in the nature of the subsequent molt occurred once more at a head capsule width of 5.1 mm. If the threshold size had been proportional to the size of the penultimate instar, the transition would have occurred at a head capsule width of about 4.3 mm.

Finally, the degree of heteromorphosis is also affected by the size of the larva (Staal, 1971). Although larvae of *Manduca* exhibit little heteromorphic development, the 5th instar larva is readily recognized by its smooth green integument, lacking the white tubercles that dot the body of the earlier instars. These tubercles are particularly evident on the head capsule of the 4th instar larva. Fifth instar larvae of intermediate sizes are also intermediate on this morphological character, showing progressively fewer and smaller tubercles as head capsule size increases. Figure 6A shows that the transition of morphology from the 4th to the 5th instar occurred at a head capsule width of 4.7 mm.

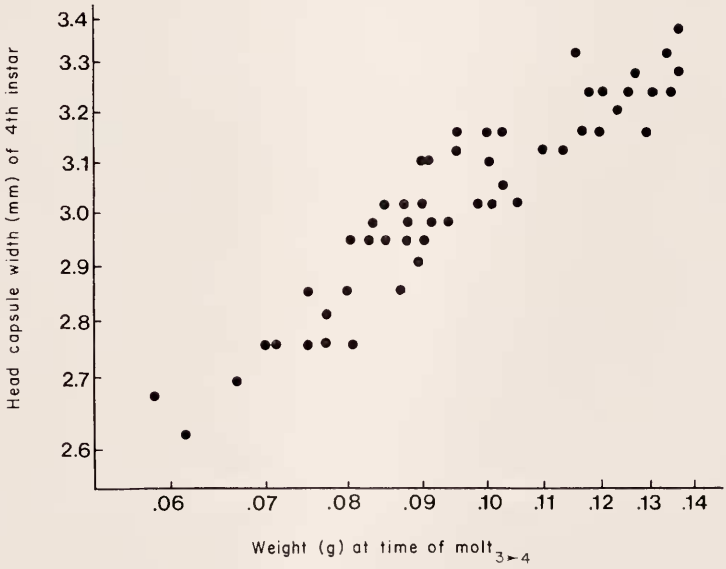

FIGURE 5. Plot of the weight at the time of molting of 3rd instar larvae starved at various weights *vs.* the head capsule width of the resulting 4th instar larva.

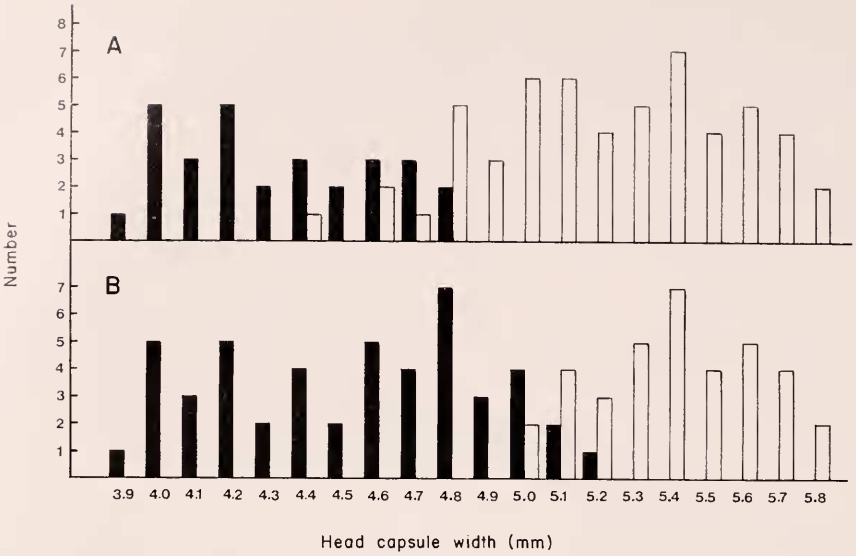

FIGURE 6. (A) Histogram showing the transition in morphology of head capsule and thoracic segments with increasing head capsule size. *White* indicates larvae possessing white tubercles typical of the 4th instar. *Black* indicates larvae with a smooth green integument typical of the 5th instar. (B) Histogram showing the number of 5th instar larvae with various head capsule sizes which pupated (white) or formed a supernumerary larval instar (black) at the succeeding molt. All larvae were derived from temporarily starved 3rd instars (*cf.* Figure 5).

DISCUSSION

According to Bounhiol (1938), larvae of Lepidoptera undergo a "period of indispensable nutrition" before they become competent to molt. In the case of *Manduca* this would be the period needed to grow to a weight of about 3 g during the 5th instar (Figure 3). Nijhout and Williams (1974a) have shown that pupation in *Manduca* occurs progressively later as larvae are starved at lower weights. The limit to the period in which the molt can occur is set by the death of the larva. Therefore, it is likely that the period of indispensable nutrition will depend on the length of time that a starved individual can survive. Since death of starved larvae appears to be largely due to dehydration, the rate of dehydration will determine the length of the period of indispensable nutrition. If the rate of dehydration were faster, the period of indispensable nutrition would be longer and *vice versa*.

When 5th instar larvae were starved at weights below 4 grams, a large percentage of the individuals transformed into larval-pupal intermediates. This was due to the fact that they had not been allowed to attain the critical weight (5 g) at which the CA are inactivated (Nijhout and Williams, 1974a, b). Consequently, the molt occurred in the presence of a substantial titer of JH.

Under the laboratory conditions described in the methods, larvae of *Manduca sexta* invariably have 5 instars. This observation suggests that the number of

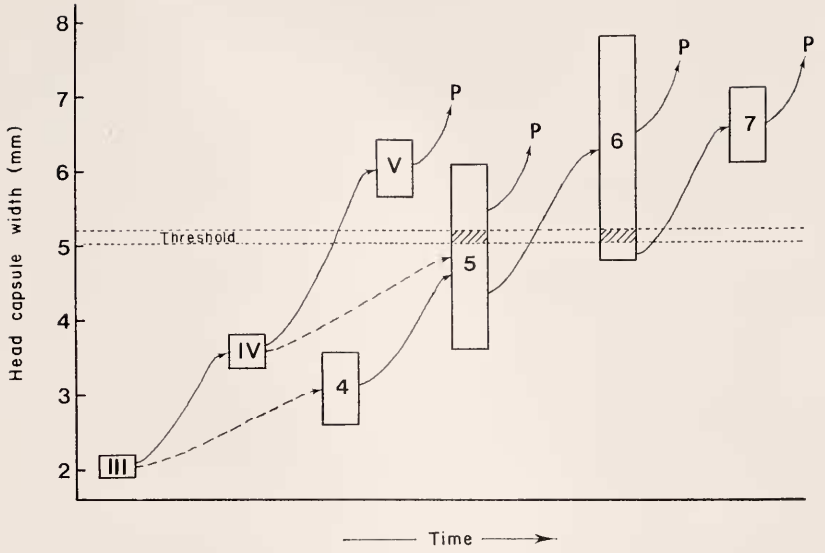

FIGURE 7. Diagrammatic summary of the experiments described under results. The vertical dimensions of the boxes indicate the range of larval head capsule widths found in each group. Roman numerals indicate larvae growing under standard laboratory conditions. Arabic numerals indicate larval instars derived from 3rd and 4th instar larvae that had been temporarily starved (dashed lines). Larvae with head capsules smaller than threshold size (5.1 mm) molt to an additional larval instar. Those with head capsules larger than threshold size pupate (P).

larval instars in this species is a determinate character and that the 5th instar is the final instar.

When 3rd or 4th instar larvae were temporarily starved and allowed to molt at a subnormal weight, the resulting 5th instar larvae were proportionally smaller, as measured by their head capsule sizes (Figures 2 and 5). The subsequent fate of these individuals depended on their absolute size. Only larvae with a head capsule above a threshold size of 5.1 mm pupated at the next molt. Smaller larvae underwent additional larval molts (Figures 4 and 6B). In a few instances the 6th instar larvae that resulted were also below threshold size for metamorphosis and formed a 7th instar (Table I). Larval-pupal intermediates were never formed in these experiments. These experiments are summarized in Figure 7. Except for size, supernumerary larval instars, as well as the pupae, that were obtained were normal and viable.

The data presented in Table I and Figure 6B indicate that the threshold size for metamorphosis does not depend on the size of the preceding instars. Rather, it appears to be a pre-coded threshold of absolute size which is not affected by the developmental history of the individual. The number of larval instars is therefore an indeterminate character. A larva does not "count instars" but rather, it continues to grow and molt until it reaches a certain threshold size. The instar in which this threshold (corresponding to a head capsule width of 5.1 mm) is reached or exceeded is then the final larval instar. The results presented above

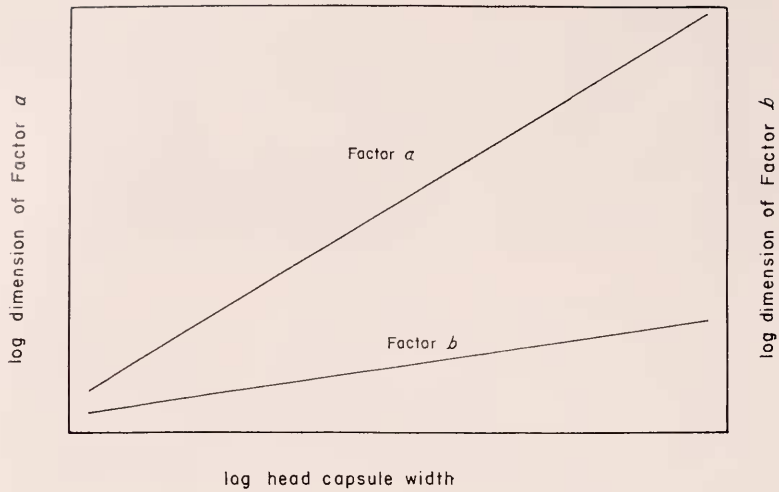

FIGURE 8. Dimension of two factors (*a* and *b*) at the time of molting versus the head capsule size of the next instar. For explanation, see text.

suggest that the commitment to metamorphose is made at the outset of the instar; possibly at the time of the molt when the new head capsule and body sizes are established.

To my knowledge, this is the first time that a threshold of absolute size has been recognized in the control of metamorphosis of an insect. The situation in *Manduca* is therefore quite different from that in *Trogoderma* where Beck (1971a, b; 1972) was able to show that metamorphosis did not depend on factors associated with size or weight. Allegret (1964) showed that larvae of *Galleria* isolated on a protein-free diet proceeded to metamorphose only if isolation occurred when the larvae exceeded a weight of about 18 mg. Larvae isolated at lower weights survived for long periods (60–100 days); during this time they failed to grow and only larval molts took place. Allegret apparently did not attach any significance to the actual dimensions of the larva at the time of isolation. His discussion of the results is restricted to the definition of a critical period during the penultimate larval instar, at which time the larva becomes committed to metamorphose. Unlike *Galleria,* larvae of *Manduca* become committed to metamorphose only at the time of the molt to the final larval instar.

The sharp transition in the nature of the subsequent molt with increasing larval size (Figure 4) suggests that the size-monitoring mechanism used by the larva is highly accurate. The question that now arises is: how can an animal assess its own size so that it may choose a radically different path of development once a pre-set threshold of *absolute* size has been reached? The simplest, if not the only, way in which an individual can monitor its own absolute size is through some form of allometry, *i.e.,* by monitoring the relative growth of one part of its body in relation to some other part or to the body as a whole. Figure 8 illustrates a possible model for such a mechanism. Consider two factors, *a* and *b* (which may be morphological or chemical), that vary allometrically with the size of the larva

but have different growth constants. In this case, for purposes of illustration, a stretch receptor mechanism could serve this function. Factor *a* could then be the distance between the attachment points of the receptor spindle, and factor *b* the unstretched length of the spindle. The ratio between these two factors continually changes as the animal grows. As the individual grows the receptor does not "keep up" and becomes progressively more stretched. Therefore, at each instar, the receptor has a higher spontaneous firing rate. It is conceivable that an individual can recognize when the firing rate exceeds a certain threshold value. The point at which this threshold value is reached is the "critical ratio of factors *a* and *b*". Since time plays no role in true allometric growth, the critical ratio of factors *a* and *b* is always reached at a particular absolute size of the individual irrespective of the time (or the number of molts) needed to reach this size. The instar in which the critical ratio of factors *a* and *b* is attained or exceeded will be the final larval instar during which the CA are shut off. At this time it is not possible to say anything about the nature of the factors that the larva uses to assess its size, or the pathway by which these factors influence the CA.

The somatic size of an individual plays a key role in guiding the pattern of development of *Manduca* in two distinct ways. First, Nijhout and Williams (1974a, b) have shown that the release of the prothroacicotropic hormone (PTTH) is inhibited in the presence of JH and that the inactivation of the CA during the final larva instar depends on the attainment of a critical weight of about 5 grams. As soon as the JH has disappeared from the hemolymph the larva proceeds to secrete PTTH and initiates the pupal molt. Consequently, the inactivation of the CA not only determines that the next molt will be a pupation but it also controls the exact timing of this event. Secondly, in the present paper I have presented evidence for the existence of another size-dependent mechanism that determines which instar is to be the final larval instar. By means of this mechanism the larva, in effect, monitors its own absolute size. Only when a sharply defined threshold of absolute size is exceeded does the first mechanism, responsible for the inactivation of the CA, enter into play.

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Summary

1. Under standard laboratory rearing conditions, caterpillars of *Manduca sexta* invariably go through 5 larval instars before pupating.

2. Evidence is presented that the head capsule width of a given instar is proportional to the weight that the individual had attained at the time that the molt to that instar occurred. Fifth-instar larvae with a large range of head capsule sizes

were produced by temporarily starving 3rd and 4th instars, thus inducing them to molt at subnormal weights.

3. Further observations on such larvae revealed that individuals with head capsules wider than 5.1 mm proceeded to pupate at the following molt whereas larvae with smaller head capsules underwent a supernumerary larval molt.

4. It was concluded that larvae of *Manduca* simply continue to grow and molt until they reach or exceed a sharply defined threshold size (corresponding to a head capsule size of 5.1 mm). The instar in which this threshold size is attained is then the final larval instar during which the *corpora allata* will be inactivated.

5. This threshold size for metamorphosis is absolute and does not depend on the prior growth history of the larva. An allometry model is presented for a mechanism by which an animal could "measure" its own absolute size.

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